Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Wogonin suppresses proliferation and invasion of skin epithelioid carcinoma cells through Notch1

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Received March 11, 2020; **Accepted** June 12, 2020; **Published** September 30, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.6.6

Abstract: The current study was carried out to investigate the role of wogonin in proliferation and invasion of skin epithelioid carcinoma cells as well as its underlying mechanisms. For this purpose, skin epithelioid carcinoma cells were treated with 0, 5, 10 and 20μmol/L wogonin for 24, 48, 72 hours. Cell proliferation was evaluated by an MTT assay. Cell invasion was assessed by the Transwell invasion assay. The Notch1 level was analyzed by RT-qPCR for mRNA and by Western blot for protein. Results showed that wogonin inhibited the proliferation and invasion of skin epithelioid carcinoma cells in a dose-dependent manner. Wogonin treatment significantly decreased the mRNA and protein levels of Notch1. Moreover, the inhibition of cell proliferation and invasion ability by wogonin treatment was dramatically attenuated after co-treatment with 20 ng/mL doxycycline, a specific Notch1 activator. In conclusion, wogonin may inhibit skin epithelioid carcinoma cell proliferation and invasion at least partially by repressing the Notch1 gene expression.

Key words: Skin cancer; Wogonin; Notch1; Proliferation; Invasion.

Introduction

Skin cancer is one type of malignant cancers arise from epithelial cells. Clinically, the most common form is skin squamous cell carcinoma (SSCC), which is also known as prickle cell carcinoma (1, 2). Skin cancer is commonly seen in body skin that vulnerable to ultraviolet radiation, mostly in facial and upper limb skin. For the past few years, the morbidity has increased with years, especially for the aged (3, 4). It shows infiltrative growth generally. One of its pathological features is the local lesion of abnormal proliferation and metastasis, and as for that, the distant metastasis can easily occur during a later period followed by poor prognosis (1, 5). Operation is currently the main therapy. However, the research and development of effective antitumor drugs are essential, to patients who lost operation opportunity, and for recurrence prevention after the operation (6-7).

Wogonin (5, 7- dihydroxy- methoxyflavone), a kind of flavonoids compound extracted from Radix astragali, has functions on specifically inducing the apoptosis of tumor cells, inhibiting tumor cell proliferation and invasion, and promoting tumor cell death with anticancer, etc (8-10). However, as for the regulating effect of wogonin on proliferation and invasion of skin epithelioid carcinoma cells, there is no literature reported. In this study, the investigation for the role of wogonin on proliferation and invasion of skin cancer cells and the potential mechanism provides an experimental basis for the treatment of skin cancer with wogonin.

Materials and Methods

Materials

Wogonin (CAS:691-47-8; HPLC≥98%) was purchased from Shanghai Traditional Chinese Medicine Standardization Research Center (Shanghai, CHINA); DMEM (Dulbecco's Modified Eagle Medium) cell culture medium, double-antibody serum and FCS (fetal calf serum) purchased from Abcam (Cambridge, MA, USA); Rabbit Anti-Human Notch1 polyclonal antibody from Abcam (Cambridge, MA, USA); β-Actin internal control antibody purchased from Cell Signaling Technology (Danvers, MA, USA); HRP-conjugated Goat Anti-Rabbit IgG from Beijing Lab Tech Biotech Company (Beijing, CHINA); Thermo26616 medium molecular Prestained Protein Marker from Thermo Fisher (Waltham, MA, USA); Super ECL Detection Reagent (NCI4106)purchased from Millipore (Billerica, MA, USA); RIPA Lysis Buffer and BCA Protein Assay Kit purchased from Wuhan Beyotime Biotechnology Co., Ltd. (Hubei, CHINA). Trizol, RT Kit and SYBR Green purchased from Takara (Tokyo, Japan).

Clinical specimen collection and cell culture

Normal skin tissue and skin cancer tissue specimens were collected from the Tissue Specimen Repository of Shaanxi Provincial People's Hospital of Xi'an since 2016-2017 and had been strictly determined by the Pathology Department. All clinical specimens used in this study have been licensed and approved by the Hospital Ethics Committee and are used for study only. Placed in DMEM complete medium (10% FCS, 100 mg/L peni-

cillin and 100 mg/L streptomycin), A431 skin epithelioid carcinoma cell line (11) was cultured in a constant temperature incubator with 5% concentration of CO2, 37 °C.

RT-PCR

The expression of Notch1 gene was detected with RT-PCR. Wash the treated cells with ice-precooled PBS (phosphate buffer saline) for 3 times. The total RNA (Ribonucleic Acid) was extracted according to the Kit instructions and A260 / A280 ratio measured. Primer sequences: β-actin Upstream primer 5'-ATGGGGAAGGTGAAGGTCG-3', downstream primer 5'-GGGTCATTGATGGCAACAATATC-3'; GAPDH Upstream primer 5'- AACTTTGGCATTGTGGAAGG-3', downstream primer 5'-ACACATTGGGGGTAGGAACA-3'; NOTCH1 Upstream primer 5'- AAGTCCCGCCGTGAAGTG-3', downstream primer 5'- ACGCCAAGGTCTGAAGGTC-3'. Reaction conditions (for both): pre-degeneration - 95°C, 3 min; 94°C, 30 s; 48°C, 30 s, 72°C, 1 min, 35 cycles; 72°C, 10 min - extension.

Western blot

The anchorage-dependent cells were washed with ice-precooled PBS for 3 times, after which RIPA Lysis buffer was added into the pellet of the cells in a 1.5ml Eppendorf tube for 30 minutes. After centrifugation at 13000 rpm for 15 min, the protein fluid was gathered and stored at -20°C. Electrophoresis was done at 80V and 120V, while the transfer was performed at 300 mA. The protein membrane was then placed in BSA (Bovine Serum Albumin) for 1h to block incubation. Later on, Rabbit Anti-Human Notch1 polyclonal antibody (1:1000; detected a band at 95kDa), Rabbit Anti-Human β-actin polyclonal antibody (1:5000; detected a band at 42kDa), Rabbit Anti-Human GAPDH polyclonal antibody (1:2000; detected a band at 42kDa) were used for 16 h-incubation. The next day, gels were washed 3 times with TBST (a mixture of tris-buffered saline and Polysorbate 20) followed by 1 h-incubation with HRP-conjugated Goat Anti-Rabbit IgG (immunoglobulin G). In the end, ECL Detection Reagent was added for exposure and analysis.

MTT assay for cell proliferation rate detection

The treated skin epithelioid carcinoma cells were digested and resuspended into a single-cell suspension, which was later placed into 96-well plates at a density of 5 x 10^3/well. A 10 μl of 0.5 mg/ml MTT was added to each well after incubation for 0, 24, 48, and 72 hours and the cells were incubated at 37°C for 4 h in dark. After removal of culture solution, the plates were added with 150 μl of DMSO (Dimethyl sulfoxide) in each well and placed on a decoloring shaker for agitation over 5min to make the crystal in well fully dissolved. The OD (optical density) value was detected with a microplate reader at 492 nm.

Transwell invasion assay

The transwell chamber was coated with 0. 1 mL of matrigel and placed in a 37 °C incubator for 1 h-incubation. The A431 skin epithelioid carcinoma cells that had been treated for 24 h with wogonin at different concentrations were digested and resuspended using serum-free DMEM culture medium and reseeded at a concentration of 5 × 10^5 cells/ml. Subsequently, the solution with resuspended cells was added into the upper chamber, while the lower chamber was filled with DMEM culture medium containing 20% FCS at 37 °C, 5% CO_2 incubator for 24 h cultivation. Afterward, we gently wipe off the cells that did not invade therefore left in the transwell chamber's upper surface using a swab. Cells gathering in the lower surface of the transwell chamber that had made an invasion were immobilized using 100% methanol and stained with hematoxylin and eosin. Examined under the microscope, 5 fields of view were taken randomly and photographed. The experiment was repeated three times.

Statistics

In this study, all the experiments were analyzed statistically using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as χ±s, t-test was used for comparison of the sample average, and one-way analysis of variance for multi-groups comparison. Tukey's HSD (honestly significant difference) test is used in conjunction with an ANOVA to find means that are significantly different from each other. α =0.05 adopted for inspection level; P<0.05 was considered statistically significant.

Results

Expression of Notch1 gene in normal tissue *vs.* skin cancer tissue

The results of RT-PCR and Western blot showed that the mRNA and protein levels of Notch1 in skin cancer tissue were significantly increased, compared to that in normal tissue (p<0.05), suggesting a possible role of Notch1 gene in the occurrence and development of skin cancer (Figure 1).

The effects of wogonin on the proliferation of skin epithelioid carcinoma cell

The results of MTT showed that the proliferation of skin epithelioid carcinoma cells A431 was dose-dependently inhibited after the intervention with wogonin, compared to control groups. The difference is considered statistically significant (p<0.05) (Figure 2).

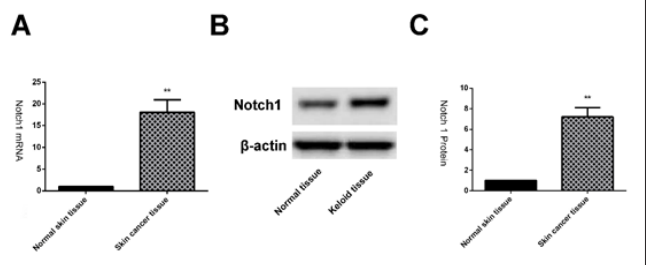

Figure 1. The mRNA and protein levels of Notch1 in normal tissue and skin cancer tissue. (A) The mRNA of Notch1 mRNA in normal tissue and skin cancer tissue by RT-qPCR; (B-C) The Western blot for Notch1 in normal tissue and skin cancer tissue by representative blots (B) and by quantification (C). **p<0.01.

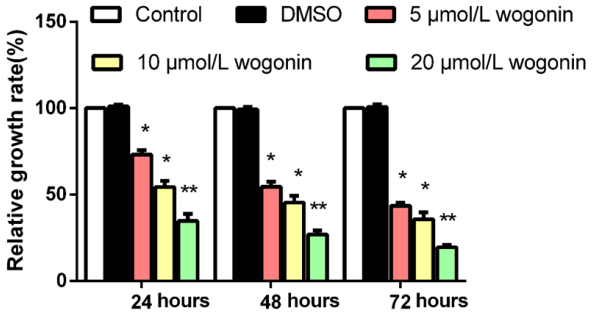

Figure 2. The effects of wogonin on the proliferation of skin epithelioid carcinoma cells. an mtt assay to show the growth of cells treated with wogonin. *p<0.05. ** p<0.01.

The Effect of wogonin on the invasiveness of skin epithelioid carcinoma cells

The results of the Transwell invasion assay show that the invasion ability of skin epithelioid carcinoma cells A431 has been dose-dependently inhibited after the intervention with wogonin, compared to the control group (p<0.05) (Figure 3).

Effects of Wogonin on the mRNA and Protein Expression of Notch1 in Skin epithelioid carcinoma cells

The results of RT-PCR and Western blot showed that the mRNA and protein levels of Notch1 in skin epithelioid carcinoma cells A431 were significantly and dose-dependently decreased after the intervention with wogonin, compared to the control group (p<0.05) (Figure 4).

The Role of Notch1 in Regulation of Skin epithelioid carcinoma cell Proliferation and Invasion

The results of the MTT assay show that the inhibiting effects of wogonin on the cell multiplication were attenuated after the use of doxycycline (20 ng/mL), a specific Notch1 gene activator. The results of the Transwell invasion assay showed that the inhibiting effects of wogonin on cell invasion were attenuated by doxycycline (20 ng/mL) (p<0.05). These data suggest that the wogonin regulates and controls the proliferation and invasion process of skin epithelioid carcinoma cells A431 possibly through inhibiting the expression of the Notch1 gene (Figure 5).

Discussion

At present, it is clinically advocated to develop individualized programs for comprehensive therapy of skin cancer. Generally, adopt regular lesionectomy, chemotherapy, radiotherapy, laser microsurgical resection, photodynamic therapy and other various methods could be selected to apply individually or in combination based on patients' age and gender, disease location, and metastatic conditions (12-13). However, patients with skin cancers who have undergone successful clinical operations still have a recurrence rate of 12%, and the 5-year metastasis rate reaches 6%. Moreover, the prognosis of skin cancer remains poor with a 5-year survival rate of 18% only (14-15). Thus, for the treatment of metastatic skin squamous cell carcinoma, the comprehensive therapy combining operation and chemotherapy drugs, such as vincristine and bleomycin, is

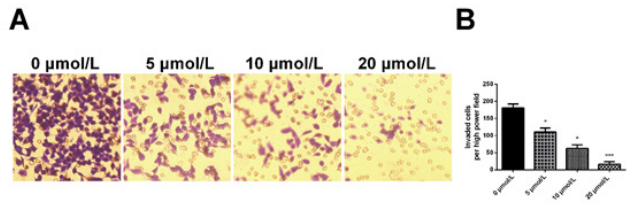

Figure 3. The effect of wogonin on the invasion of skin epithelioid carcinoma cell. (A-B) Transwell assay to detect the invasion of A431 cells, shown by representative images (A) and by quantification (B). *p<0.05. ***p<0.001.

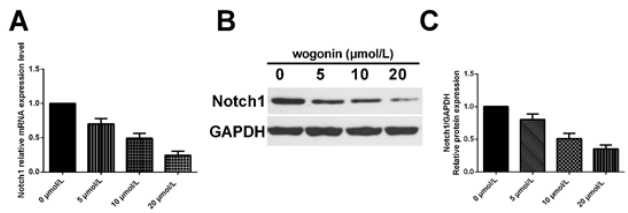

Figure 4. Effects of wogonin on the mRNA and protein levels of Notch1 in skin epithelioid carcinoma cells. (A) RT-qPCR for Notch1 mRNA levels; (B-C) Western blot analysis of Notch1 protein, shown by representative blots (B) and by quantification (C). **p<0.01.

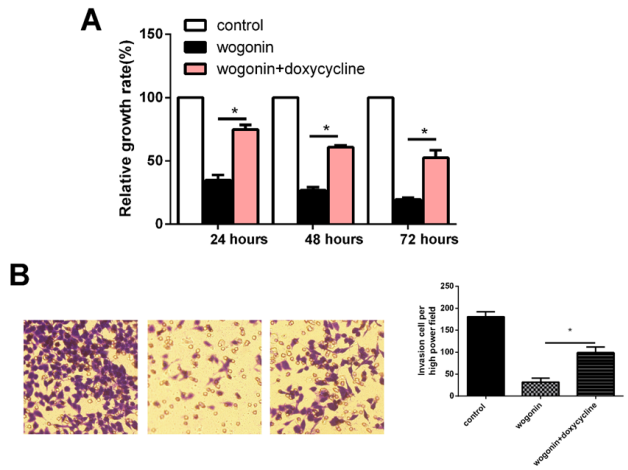

Figure 5. The role of the Notch1 gene in the regulation of skin epithelioid carcinoma cell proliferation and invasion. (A) The effects of doxycycline on the proliferation of skin epithelioid carcinoma cells. (B-C) Transwell assay for the invasion of A431 cells, shown by representative images (B) and by quantification (C). The left panel in B: untreated control. The middle panel in B: wogonin. The right panel in B: wogonin+doxycycline. *p<0.05.

still commonly applied (16-17). However, the efficacy of chemotherapy drugs could not meet expectations yet and in addition, it brings toxic and side effects to normal cells (18). Therefore, it is urgent for clinical and basic medical research to develop new medicine for the treatment of skin squamous cell carcinoma.

Wogonin is an active ingredient mainly from radix astragali and barbed skullcap herb and is a kind of common natural flavonoid compound. It has the advantages of extensive sources, low cost, no side effects, etc. In recent years, the anti-tumor effect of Wogonin has been reported (19-21). In hepatocellular carcinoma, wogonin could inhibit the expression of matrix metalloproteinase

- 9 and the translocation and invasion ability of tumor cells (22). Liu etc. reported that the wogonin could induce the apoptosis of hepatoma cells by regulating and controlling NF-κB (nuclear factor-κB), EGFR (epidermal growth factor receptor) and EGFR's downstream pathway - ERK/AKT (extracellular signal-regulated kinase/protein kinase B, AKT), inhibiting proliferation and invasion of hepatoma carcinoma cell (23). In addition, the wogonin could suppress the translocation and invasion of malignant melanoma B16-F10 cells through a blocking signal pathway which is mediated by RAS (24). In cervical cancer cells, wogonin may play a role in against cancer by promoting the apoptosis of tumor cells by the inhibition of E6 and E7 expression.

Notch1 is a highly conservative cell transmembrane glycoprotein and has various functions in regulating cell proliferation, apoptosis, invasion and metastasis and other biological processes. Not only is the Notch1 gene involved in the growth and development of normal human cells, but also is it related to the development of multiple malignant tumors. Notch1 gene is activated after the binding of its receptors with ligands. The intracellular active functional fragments are subsequently released from acceptor molecules directly to the cell nucleus, to regulate and control the expression of downstream target genes and further inhibit the expression of cell cycle-related regulatory proteins to suppress cell proliferation and terminate cell cycles. Previous studies have shown abnormally high expressions of Notch1 expression in skin cancer tissue, and the Notch1 may accelerate the invasion and metastasis of cancer cells by suppressing E-cadherin's expressions. Recently, it was shown that wogonin could induce retinal neurons shaped differentiation of mesenchymal stem cells by inhibiting the Notch1 gene's expressions. Based on the above findings, we hypothesized that wogonin may restrain the proliferation and invasion of skin epithelioid carcinoma cells after its suppression on the Notch1 gene's expressions and thus tested it in the current study.

In this study, firstly we found the expression of the Notch1 gene in skin cancer tissue had significantly increased compared to normal skin tissue, which suggests that the occurrence and development of skin cancer involve the abnormal activation of the Notch1 signal pathway. Second, we showed the dose-dependent suppressive effect of wogonin on the proliferation and invasion ability of A431 skin epithelioid carcinoma cells. Then we studied the underlying molecular mechanism. We found that the activity of the Notch1 gene was dose-dependently and significantly inhibited by wogonin, suggesting the requirement for Notch1 in the wogonin-mediated suppression of growth and metastasis of skin epithelioid carcinoma cells. To clarify the exact role that the Notch1 gene plays in the inhibition of wogonin on the proliferation and invasion of skin epithelioid carcinoma cell, we used the Notch1 gene activator, doxycycline, to simulate Notch1 gene's expression and further examined its effects on cell proliferation and invasion. As the results showed, the inhibitory effects of wogonin on the proliferation and invasion of skin epithelioid carcinoma cells were attenuated by the use of doxycycline, suggesting the Notch1 signal pathway indeed plays an important role. These data suggest that wogonin may inhibit the abnormal proliferation and distant invasion and metastasis of skin epithelioid carcinoma cells through regulating transcriptional activation of the downstream target gene by suppressing the Notch1 signal pathway. Gene expression and control have always been of particular importance in molecular and genetic research, and attention to this important issue in the future could be effective in treating many human diseases, failures, and disorders (25-37).

This study suggests that the wogonin may inhibit the abnormal proliferation and invasion ability in vitro of skin epithelioid carcinoma cells mainly by Notch1. This study provides new ideas for further investigations on the role of wogonin in the prevention and treatment of skin cancer with a focus on the underlying molecular mechanisms.

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